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BZLF1, an Epstein–Barr virus immediate–early protein, induces p65 nuclear translocation while inhibiting p65 transcriptional function

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Abstract

We have previously demonstrated that the Epstein–Barr virus immediate–early BZLF1 protein interacts with, and is inhibited by, the NF-κB family member p65. However, the effects of BZLF1 on NF-κB activity have not been intensively studied. Here we show that BZLF1 inhibits p65-dependent gene expression. BZLF1 inhibited the ability of IL-1, as well as transfected p65, to activate the expression of two different NF-κB-responsive genes, ICAM-1 and IκB-α. BZLF1 also reduced the constitutive level of IκB-α protein in HeLa and A549 cells, and increased the amount of nuclear NF-κB to a similar extent as tumor necrosis factor-alpha (TNF-α) treatment. In spite of this BZLF1-associated increase in the nuclear form of NF-κB, BZLF1 did not induce binding of NF-κB to NF-κB responsive promoters (as determined by chromatin immunoprecipitation assay) in vivo, although TNF-α treatment induced NF-κB binding as expected. Overexpression of p65 dramatically inhibited the lytic replication cycle of EBV in 293-EBV cells, confirming that NF-κB also inhibits BZLF1 transcriptional function. Our results are consistent with a model in which BZLF1 inhibits the transcriptional function of p65, resulting in decreased transcription of IκB-α, decreased expression of IκB-α protein, and subsequent translocation of NF-κB to the nucleus. This nuclear translocation of NF-κB may promote viral latency by negatively regulating BZLF1 transcriptional activity. In situations where p65 activity is limiting in comparison to BZLF1, the ability of BZLF1 to inhibit p65 transcriptional function may protect the virus from the host immune system during the lytic form of infection.

Keywords: Epstein–Barr virus; p65; BZLF1; Transcription

Introduction

Epstein–Barr virus (EBV) is a human γ-herpesvirus that persistently infects the majority of the world population. Primary infection with EBV occurs in the oropharynx via saliva exchange and may lead to the development of the syndrome infectious mononucleosis (Kieff and Rickinson, 2001; Rickinson and Kieff, 2001). Additionally, EBV is associated with an increasing number of human malignancies, of both lymphoid and epithelial origin, including nasopharyngeal carcinoma, gastric carcinoma, Burkitt's lymphoma, post-transplant lymphoproliferative disease, and Hodgkin's lymphoma (Kieff and Rickinson, 2001; Rickinson and Kieff, 2001).

EBV lytic infection is initiated by the expression of two immediate–early (IE) genes, *BZLF1* and *BRLF1* (Chevallier-Greco et al., 1986; Countryman and Miller, 1985; Hardwick et al., 1992; Ragoczy et al., 1998; Rooney et al., 1989; Takada et al., 1986; Urier et al., 1989; Zalani et al., 1996). The *BZLF1* gene product (BZLF1, Zta, ZEBRA, EB1) is a member of the basic leucine zipper (b-zip) family of DNA binding proteins that binds to AP-1-like sites, termed Z response elements (ZREs), present in EBV IE and early gene promoters (Chang et al., 1990; Farrell et al.,

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1989; Flemington and Speck, 1990; Flemington et al., 1992; Lieberman et al., 1989). BZLF1 functions as a transcriptional transactivator and, along with the other EBV IE gene *BRLF1*, induces the lytic cascade of viral gene expression (Feederle et al., 2000).

The NF-κB family of transcription factors are critical for inducible expression of a wide variety of genes involved in immunity and inflammation (Baldwin, 2001; Ghosh et al., 1998). In unstimulated cells, the majority of NF-κB resides in the cytoplasm in association with the IκB family of inhibitory molecules, which mask the nuclear localization signals of NF-κB (Ghosh and Karin, 2002). The most abundant form of NF-κB in most cells is a heterodimer of p65/RelA and p50. NF-κB signaling is activated by a variety of stimuli including cytokines and growth factors that activate the IκB kinase complex (IKK). Once activated, IKK phosphorylates IκB family members at conserved serine residues (DiDonato et al., 1997; Mercurio et al., 1997; Woronicz et al., 1997; Zandi et al., 1997). This phosphorylation results in ubiquitin-mediated degradation of IκBs by the proteasome (Karin and Ben-Neriah, 2000). Degradation of IκB releases NF-κB to translocate to the nucleus where it binds kB enhancer elements and modulates gene expression. NF-κB also strongly activates the IκB-α promoter, allowing IκB-α to be regenerated in cells and thus terminate the NF-κB signaling cascade (Arenzana-Seisdedos et al., 1995; Brown et al., 1993; Chiao et al., 1994; Sun et al., 1993).

Activation of NF-κB is a feature of many viral infections (Mogensen and Paludan, 2001). NF-κB activation during viral infection is believed to be a protective response of the host to the viral pathogen. Therefore, many viruses have evolved distinct strategies to control the activity of NF-κB to evade the immune response (Hiscott et al., 2001; Santoro et al., 2003). Additionally, viruses may modulate the NF-κB pathway to enhance viral replication or prevent virus-induced apoptosis (Hiscott et al., 2001; Santoro et al., 2003).

BZLF1 and p65 have been previously shown to directly interact, and co-expression of p65 inhibited BZLF1 transactivation of several EBV lytic gene promoters (Gutsch et al., 1994). However, the effects of BZLF1 on NF-κB activity have not been extensively studied. Two studies suggested that BZLF1 expression in B cells and T cell lines decreased transcription of NF-κB reporter genes (Dreyfus et al., 1999; Keating et al., 2002). In contrast, another study reported that a DNA binding-defective BZLF1 activated the expression of an NF-κB-responsive reporter construct (Flemington et al., 1994). Thus, the effect of BZLF1 on NF-κB transcriptional function remains unclear, particularly in regard to its effect in the context of the intact cellular genome.

Here, we show that BZLF1 inhibits p65-dependent gene expression induced by either the IL-1 cytokine or transfected p65 in the context of the cellular genome. BZLF1 decreases constitutive transcription of the p65-responsive IκB-α gene in HeLa and A549 cells, resulting in decreased IκB-α protein expression. Consistent with its ability to inhibit IκB-α expression, BZLF1 expression also induces nuclear translocation of NF-κB, similar to the effect of TNF-α treatment. In contrast to the effect of TNF-α, however, chromatin immunoprecipitation assays demonstrated that the nuclear NF-κB induced by BZLF1 did not bind to the IκB-α or ICAM-1 promoters in vivo. Overexpression of p65 inhibited the lytic replication cycle of EBV in 293-EBV cells, confirming previous studies suggesting that NF-κB inhibits BZLF1 transcriptional function. Our results are consistent with a model in which BZLF1 inhibits p65-mediated transcription, which results in decreased IκB-α expression and consequently increases NF-κB translocation to the nucleus. The ability of BZLF1 to indirectly promote nuclear translocation of NF-κB through its effects on IκB-α may promote viral latency by negatively regulating BZLF1 transcriptional activity. In contrast, in situations where p65 activity is limiting in comparison to BZLF1, the ability of BZLF1 to inhibit p65 transcriptional function may protect the virus from the host immune system during the lytic form of infection.

Results

BZLF1 inhibits p65-induced gene expression

The BZLF1 gene product has been previously shown to interact with, and be inhibited by, the NF-κB family member p65 (Gutsch et al., 1994). However, the effects of BZLF1 on NF-κB activity have not been thoroughly investigated, particularly in regard to the ability of p65 to activate genes in the context of the cellular genome. To determine if BZLF1 modulates p65-mediated gene expression, HeLa cells were transfected with a FLAG-tagged p65 expression vector (FLAG-p65) together with a control plasmid or a plasmid expressing BZLF1. The ability of transfected p65 to induce expression of two different NF-κB responsive genes (ICAM-1 and IκB-α) in the context of the cellular genome was evaluated by immunoblot analysis. As expected, transfection of HeLa cells with FLAG-p65 induced ICAM-1 expression (Fig. 1A) as well as expression of IκB-α (Fig. 1A). However, co-transfection of BZLF1 with FLAG-p65 greatly inhibited p65-induced ICAM-1 expression, as well as p65-induced IκB-α expression (Fig. 1A). In contrast to the effects observed in the intact cellular genome, BZLF1 actually enhanced the ability of transfected p65 to active NF-κB reporter constructs that contained the consensus NF-κB binding motif, or the IκB-α promoter, linked to the secretory alkaline phosphatase gene (Figs. 1B, C). Similar results were obtained using luciferase-based NF-κB reporter constructs, indicating that these effects were not reporter gene specific (data not shown). Immunoblot analysis confirmed that the level of transfected p65 was similar or actually greater in the presence of co-transfected BZLF1 (Fig. 1B). These results suggest that in the context

of the intact cellular genome, BZLF1 inhibits the expression of at least a subset of NF-κB responsive genes, and that this effect cannot be adequately modeled using reporter gene constructs.

Previous studies have demonstrated that CBP functionally interacts with p65 and enhances p65-mediated transactivation (Gerritsen et al., 1997). Similarly, BZLF1 also directly interacts with CBP (Adamson and Kenney, 1999; Chen et al., 2001; Deng et al., 2003; Zerby et al., 1999).

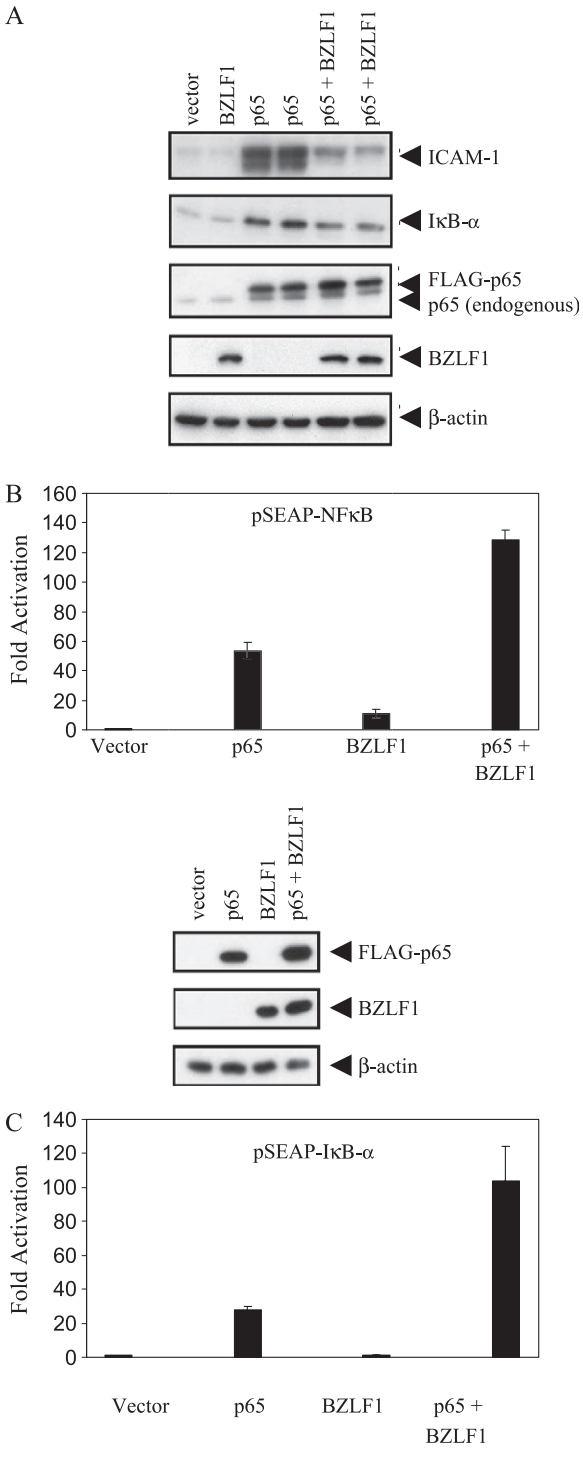

Fig. 1. BZLF1 inhibits p65-induced ICAM-1 and IκB-α expression. (A) HeLa cells were transfected with 500 ng each of vector, BZLF1, or FLAG-tagged p65 expression plasmids. At 48 h post-transfection, immunoblot analyses were performed with anti-IκBα, anti-ICAM-1, anti-BZLF1, anti-p65, and anti-β-actin antibodies. (B) HeLa cells were transfected with 500 ng each of vector, BZLF1, or FLAG-tagged p65 expression plasmids together with 500 ng of pSEAP-NFκB reporter plasmid. At 48 h post-transfection, SEAP activity was assayed in tissue culture supernatants. Immunoblot analyses were performed with anti-FLAG, anti-BZLF1, and anti-β-actin antibodies. (C) HeLa cells were transfected with 500 ng each of vector, BZLF1, or FLAG-tagged p65 expression plasmids together with 500 ng of pSEAP-IκB-α reporter plasmids. At 48 h post-transfection, SEAP activity was assayed in tissue culture supernatants.

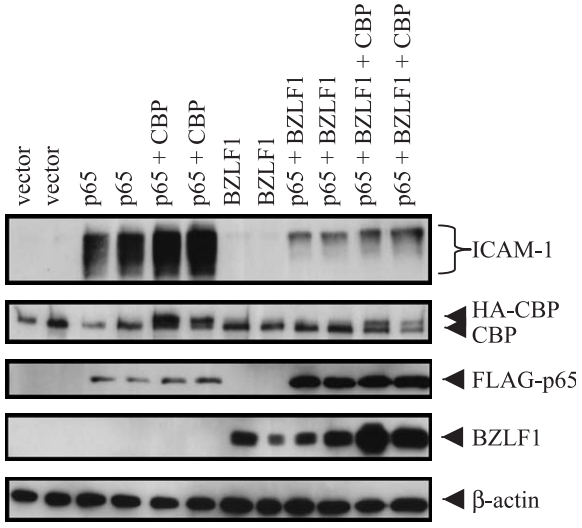

Fig. 2. BZLF1 inhibition of p65 function is not reversed by CBP. HeLa cells were transfected with 500 ng of a HA-tagged CBP expression plasmid along with 500 ng of vector, FLAG-tagged p65, or BZLF1 expression plasmids. Immunoblot analyses were performed with anti-CBP, anti-ICAM-1, anti-BZLF1, anti-FLAG antibodies, and anti-β-actin antibodies.

Thus, BZLF1 may inhibit p65-mediated gene expression by competing for limiting amounts of cellular CBP. To test this hypothesis, HeLa cells were transfected with FLAG-p65, HA-CBP, and BZLF1 expression plasmids and immunoblot analysis was performed for ICAM-1 expression. Although co-transfection of HA-CBP enhanced p65-induced ICAM-1 expression, the ability of BZLF1 to inhibit p65 induction of ICAM-1 was not affected by overexpression of CBP (Fig. 2). Thus, competition for limiting amounts of CBP in the cell cannot completely explain the BZLF1-mediated inhibition of p65-dependent transcription in the context of the intact cellular genome.

To determine which domains of the BZLF1 protein are required for inhibition of p65 function, HeLa cells were transfected with FLAG-p65 together with plasmids encoding wild-type BZLF1, BZLF1 carrying a point mutation that abrogates the ability of BZLF1 to bind DNA (ZA185K), and BZLF1 deleted for amino acids 24–86, which removes the BZLF1 transactivation domain (ZΔTA). As shown in Fig. 3A, wild-type BZLF1, ZA185K, and

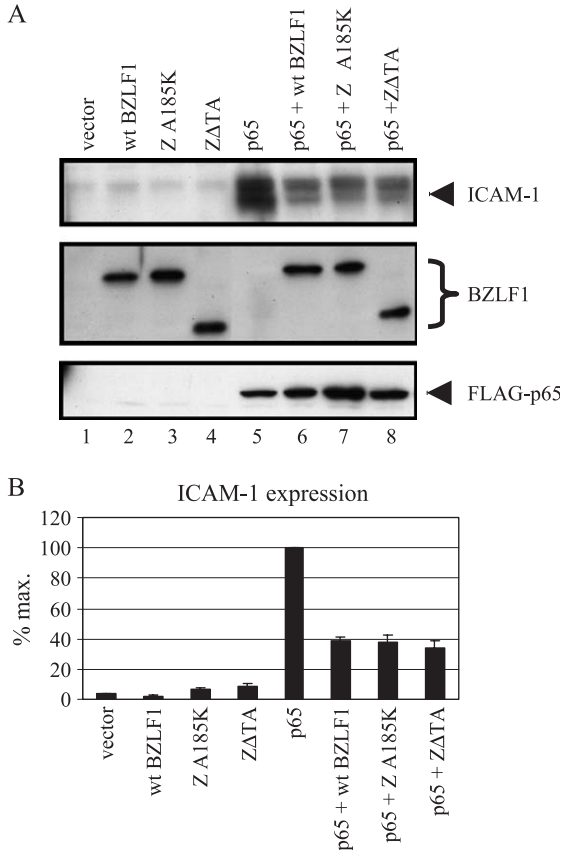

Fig. 3. BZLF1 inhibition of p65-mediated gene expression does not require the BZLF1 transactivation domain or BZLF1 DNA binding. (A) HeLa cells were transfected with 500 ng each of FLAG-tagged p65 expression plasmid and various wild-type and mutant BZLF1 expression plasmids. Immunoblot analyses were performed with anti-ICAM-1, anti-BZLF1, and anti-FLAG antibodies. (B) ICAM-1 immunoblots from two independent experiments were quantitated using the ImageJ v1.32 program (NIH) and graphed with p65-induced ICAM-1 expression set at 100%.

ZΔTA prevented ICAM-1 expression induced by transfection of FLAG-p65. Quantitation of immunoblots from two independent experiments by ImageJ (NIH) analysis confirmed that BZLF1, ZA185K, and ZΔTA significantly inhibited p65-induced ICAM-1 expression (Fig. 3B). A mutant form of BZLF1 with a deletion of the entire dimerization domain (ZΔDIM), which is required for the direct interaction between BZLF1 and p65 (Gutsch et al., 1994), as well as BZLF1 homodimerization, was also tested for its ability to inhibit p65-mediated gene induction. Co-transfection of ZΔDIM did not inhibit p65-induced gene expression (data not shown); however, we found that this particular mutant is consistently expressed at lower levels than wild-type and other mutant forms of BZLF1 making the results difficult to interpret. These findings suggest that the ability of BZLF1 to inhibit p65-induced gene expression does not require the BZLF1 transactivation domain or BZLF1 DNA binding, making it unlikely that BZLF1 indirectly inhibits p65 function by inducing expression of a cellular gene product that inhibits p65 transactivation.

BZLF1 inhibits IL-1 induced ICAM-1 expression

The ability of BZLF1 to inhibit gene expression induced by direct p65 transfection prompted us to test whether BZLF1 also inhibits gene expression induced by cytokines that signal via NF-κB activation. To test this hypothesis, HeLa cells were mock-infected or infected with adenovirus vectors expressing either the LacZ gene (AdlacZ) or the BZLF1 gene (AdZ), and at 30 h post-infection cells were unstimulated or stimulated with TNF-α, LT-α, or IL-1. In mock-infected or AdlacZ-infected HeLa cells, stimulation with TNF-α, LT-α, or IL-1 induced expression of ICAM-1 (Fig. 4A). As we have previously shown, BZLF1 inhibits multiple cellular responses to TNF-α, a major inducer of NF-κB, by down-regulation of the cellular receptor for TNF-α, TNF-R1 (Morrison et al., 2004). Consistent with these previous findings, expression of BZLF1 inhibited ICAM-1 expression induced by TNF-α and LT-α, cytokines that utilize TNF-R1. However, BZLF1 also inhibited ICAM-1 expression induced by IL-1 stimulation, which does not depend upon the presence of TNF-R1. To determine whether the upstream components of the IL-1 signaling pathway are functional in AdZ-infected cells, the ability of IL-1 to induce p65 phosphorylation on serine 536 (which occurs upstream of p65 translocation) (Sizemore et al., 1999, 2002; Yang et al., 2003) was examined using a phospho-specific p65 (serine 536) antibody (Fig. 4B). As expected, treatment with either TNF-α or IL-1 stimulated

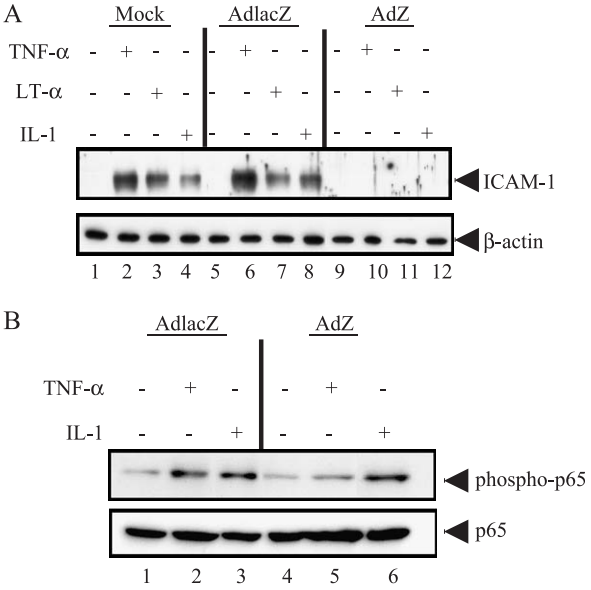

Fig. 4. BZLF1 inhibits IL-1 induced ICAM-1 expression. (A) HeLa cells were mock-infected or infected with adenovirus expressing LacZ (AdlacZ) or BZLF1 (AdZ) (moi of 30). At 30 h post-infection, cells were unstimulated or stimulated with 10 ng/ml TNF-α, 10 ng/ml LT-α, or 10 ng/ml IL-1 for 12 h. Immunoblot analysis was performed with anti-ICAM-1 and anti-β-actin antibodies. (B) HeLa cells were infected with AdlacZ or AdZ (moi 30). At 48 h post-infection, cells were unstimulated or stimulated with 10 ng/ml TNF-α or 10 ng/ml IL-1 for 10 min. Immunoblot analysis was performed with anti-phospho-p65 (serine 536) and anti-p65 antibodies.

phosphorylation of p65 in AdlacZ-infected cells (Fig. 3B), but TNF-α did not stimulate p65 phosphorylation in AdZ-infected cells because these cells do not express TNF-R1. In contrast to the effect of TNF-α, treatment of AdZ-infected cells with IL-1 resulted in phosphorylation of p65. Taken together, these results suggest that upstream components of the IL-1 signaling pathway are intact in AdZ-infected cells, and that BZLF1 inhibits IL-1 induced ICAM-1 expression downstream of NF-κB activation.

BZLF1 decreases IκB-α RNA expression

To determine if the ability of BZLF1 to prevent p65-mediated induction of IκB-α (Fig. 1A) is due to a decrease in IκB-α message, we examined the effect of AdZ on IκB-α RNA expression. Northern blot analysis on total RNA demonstrated decreased expression of IκB-α RNA in AdZ-infected HeLa cells compared to cells infected with a control adenovirus and mock-infected cells in the presence or absence of TNF-α (Fig. 5). In contrast, the level of GAPDH RNA was unaffected. Similar results were obtained in A549 cells (data not shown). These results suggest that BZLF1 dramatically decreases the level of IκB-α message in epithelial cells.

BZLF1 decreases IκB-α protein expression

To further investigate the effect of BZLF1 expression on IκB-α, HeLa cells were mock-infected or infected with AdlacZ or AdZ, and immunoblot analysis was performed to examine expression of p65 and its endogenous inhibitor IκB-α in the presence or absence of TNF-α. Infection of HeLa cells with AdZ did not affect the total level of p65 expression (Fig. 6). As predicted by the Northern blot analysis in Fig. 5, IκB-α protein expression was greatly decreased in AdZ-infected cells. The AdZ-mediated decrease in IκB-α protein was as great or greater than that induced by 30 min of TNF-α stimulation (Fig. 6), which is known to induce IκB-α degradation.

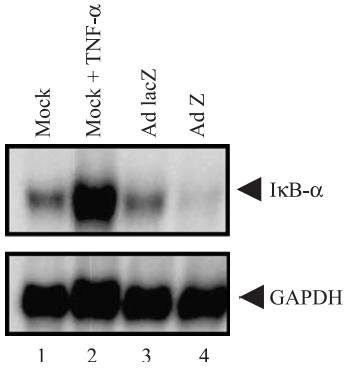

Fig. 5. BZLF1 decreases IκB-α RNA level. HeLa cells were mock-infected or infected with AdlacZ or AdZ (moi 30). Total RNA was harvested at 48 h post-infection and analyzed by Northern blot analysis using radiolabeled probes for IκB-α (upper panel) or GAPDH (lower panel).

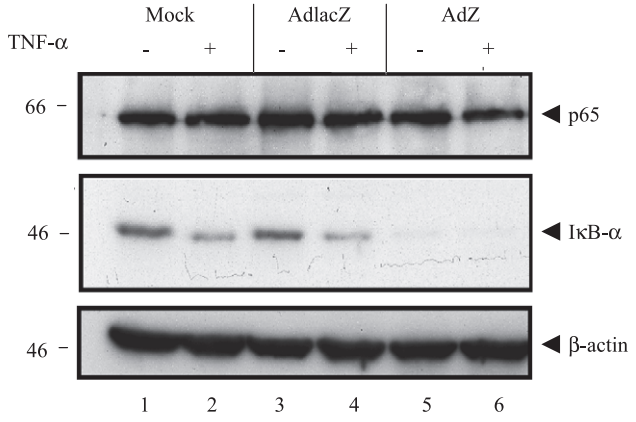

Fig. 6. IκB-α is decreased in cells expressing BZLF1. HeLa cells were mock-infected or infected with AdlacZ or AdZ (moi 30). At 48 h post-infection, cells were treated with 10 ng/ml TNF-α for 30 min and extracts were analyzed by immunoblot analysis for p65 (upper panel), IκB-α (middle panel), and β-actin (lower panel).

IκB-α protein is commonly regulated by phosphorylation-dependent ubiquitin-mediated degradation by the proteasome (Karin and Ben-Neriah, 2000). To test whether AdZ enhanced proteasomal-mediated degradation of IκB-α, HeLa cells were infected with AdlacZ or AdZ, treated with DMSO or 50 μM MG-132 for 4, 6, and 10 h, and extracts analyzed by immunoblot analysis for IκB-α. IκB-α expression remained decreased in AdZ-infected cells treated with the proteasome inhibitor (Fig. 7). Immunoblot analysis for p53 demonstrated accumulation of this protein in groups treated with MG-132, confirming that the proteasome inhibitor was functional. These results indicate that AdZ decreased IκB-α expression occurs mainly (if not entirely) through a decrease in IκB-α message.

BZLF1 induces accumulation of nuclear NF-κB

NF-κB is retained in the cytoplasm by its direct interaction with IκB-α. Thus, the finding that BZLF1 expression rather dramatically reduces IκB-α expression in HeLa cells suggested that BZLF1 may also induce

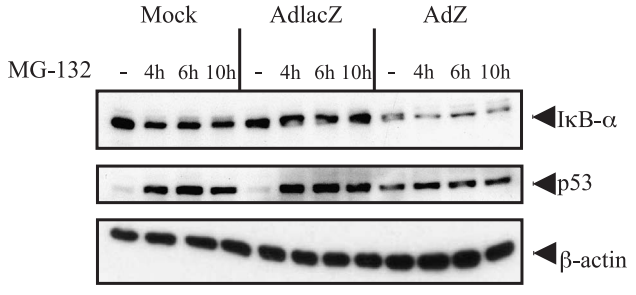

Fig. 7. BZLF1-mediated IκB-α decrease is not reversed by proteasome inhibition. HeLa cells were infected with AdlacZ or AdZ (moi 30). At 40 h post-infection, cells were treated with DMSO or 50 μM MG-132 for the times indicated, and extracts were analyzed by immunoblot analysis for IκB-α, p53, and β-actin.

NF-κB nuclear translocation. To examine the cellular location of NF-κB in the presence of BZLF1, HeLa cells were either mock-infected, infected with AdlacZ, or infected with AdZ, and at 24 and 36 h post-infection, cells were harvested and fractionated into their cytoplasmic and nuclear compartments. As shown in Fig. 8, AdZ induced nuclear accumulation of p65 at both 24 and 36 h post-infection. Significantly, the AdZ-induced nuclear accumulation of p65 at 36 h post-infection was similar to the level of p65 nuclear accumulation following 30 min of TNF-α stimulation. Immunoblot analysis confirmed that IκB-α expression was decreased in the cytoplasm of AdZ-infected cells at both 24 and 36 h (data not shown).

Electrophoretic mobility shift assays (EMSA) were performed to further confirm that BZLF1 induces nuclear NF-κB in HeLa cells. Nuclear extracts were prepared from HeLa cells infected with AdlacZ or with AdZ at 36 h post-infection, or from AdlacZ-infected cells treated with TNF-α for 30 min. As expected, nuclear extracts from TNF-α treated (AdlacZ-infected) cells had greatly increased binding to a labeled probe containing the NF-κB binding site from the MHC class I promoter (Fig. 9A), the IκB-α promoter (Fig. 9B), and the ICAM-1 promoter (Fig. 9B) in comparison to the AdlacZ-infected cells not treated with TNF-α. However, even in the absence of TNF-α treatment, nuclear extracts prepared from the AdZ-infected cells had significant binding to all three NF-κB-site containing probes (Figs. 9A, B). Similar to the TNF-α-induced binding complex, the AdZ-induced complex was supershifted with both anti-p65 and anti-p50 antibodies (Fig. 9A), confirming that the complex represents NF-κB. EMSA analysis with a labeled probe containing the c/EBP consensus site demonstrated similar binding of c/EBP-β (Fig. 9C) (the major c/EBP isoform found in the nucleus of HeLa cells), confirming that the increased binding observed in nuclear extracts from AdZ-infected cells was specific for NF-κB (Fig. 9C). The BZLF1 protein also bound the c/EBP consensus site probe (Fig. 9C), confirming expression of BZLF1 in these extracts. These results suggest that BZLF1 expression in HeLa cells results in nuclear translocation of NF-κB, similar to that observed following TNF-α treatment.

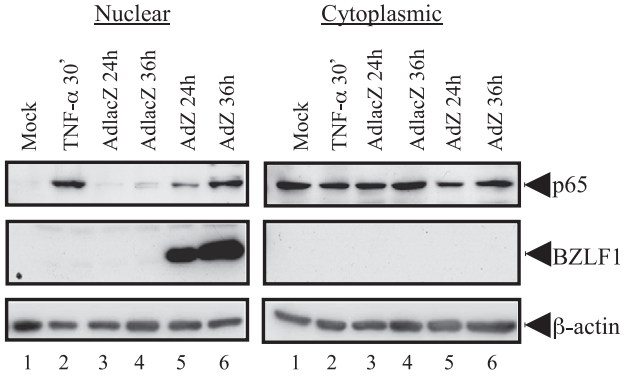

Fig. 8. BZLF1 induces nuclear accumulation of p65. HeLa cells were infected with AdlacZ or AdZ (moi 30), and nuclear and cytoplasmic extracts were analyzed by immunoblot analysis for p65, BZLF1, and β-actin.

BZLF1 inhibits NF-κB binding to cellular promoters in the context of the intact cellular genome

The previous results indicate that BZLF1 expression in HeLa cells results in increased nuclear NF-κB binding activity as measured by EMSA, yet paradoxically decreases the ability of transfected p65, or IL-1 treatment, to activate NF-κB-dependent transcription in the context of the intact cellular genome. Therefore, we examined the effect of BZLF1 on NF-κB DNA binding to NF-κB responsive promoters in vivo using chromatin immunoprecipitation (ChIP) analysis. ChIP analysis detected recruitment of p65 and p50 to the IκB-α and ICAM-1 promoters in AdlacZ-infected HeLa cells following TNF-α stimulation, as expected (Fig. 10A). The HSP-70 control showed that this TNF-α-dependent recruitment was specific to NF-κB regulated promoters (Fig. 10A). In contrast, in AdZ-infected HeLa cells, we were unable to detect p65 or p50 recruitment to either the IκB-α or ICAM-1 promoters, although the effect of BZLF1 on IκB-α protein in this experiment was at least as great as the TNF-α effect (Fig. 10B). PCR using serial dilutions of input DNA confirmed an equal level of starting material in each experimental condition (data not shown). These results suggest that BZLF1 inhibits at least a subset of NF-κB-dependent gene expression by reducing the ability of NF-κB to bind to NF-κB responsive promoters in the context of the intact cellular genome.

p65 overexpression inhibits BZLF1-mediated lytic cycle induction in 293 cells

Although it has previously been demonstrated that p65 overexpression inhibits the ability of BZLF1 to transactivate reporter constructs containing the promoters of the EBV early genes BHLF1 or BMRF1 (Gutsch et al., 1994), the effect of p65 on BZLF1 function in the context of the intact viral genome has not been well studied. To determine if p65 expression inhibits the ability of BZLF1 to induce the complete EBV lytic replication cycle, 293 cells carrying the EBV genome (Feederle et al., 2000) were transfected with an expression vector encoding BZLF1 in the presence or absence of co-transfected p65. At 48 h post-transfection, cell lysates were harvested and cell culture supernatants were collected, centrifuged, and filtered. As shown by immunoblot analysis in Fig. 11A, transfected BZLF1 alone induced expression of the other immediate–early gene product, BRLF1, and the EBV early gene product, BMRF1, in 293-EBV cells. The ability of BZLF1 to induce expression of either BRLF1 or BMRF1 was

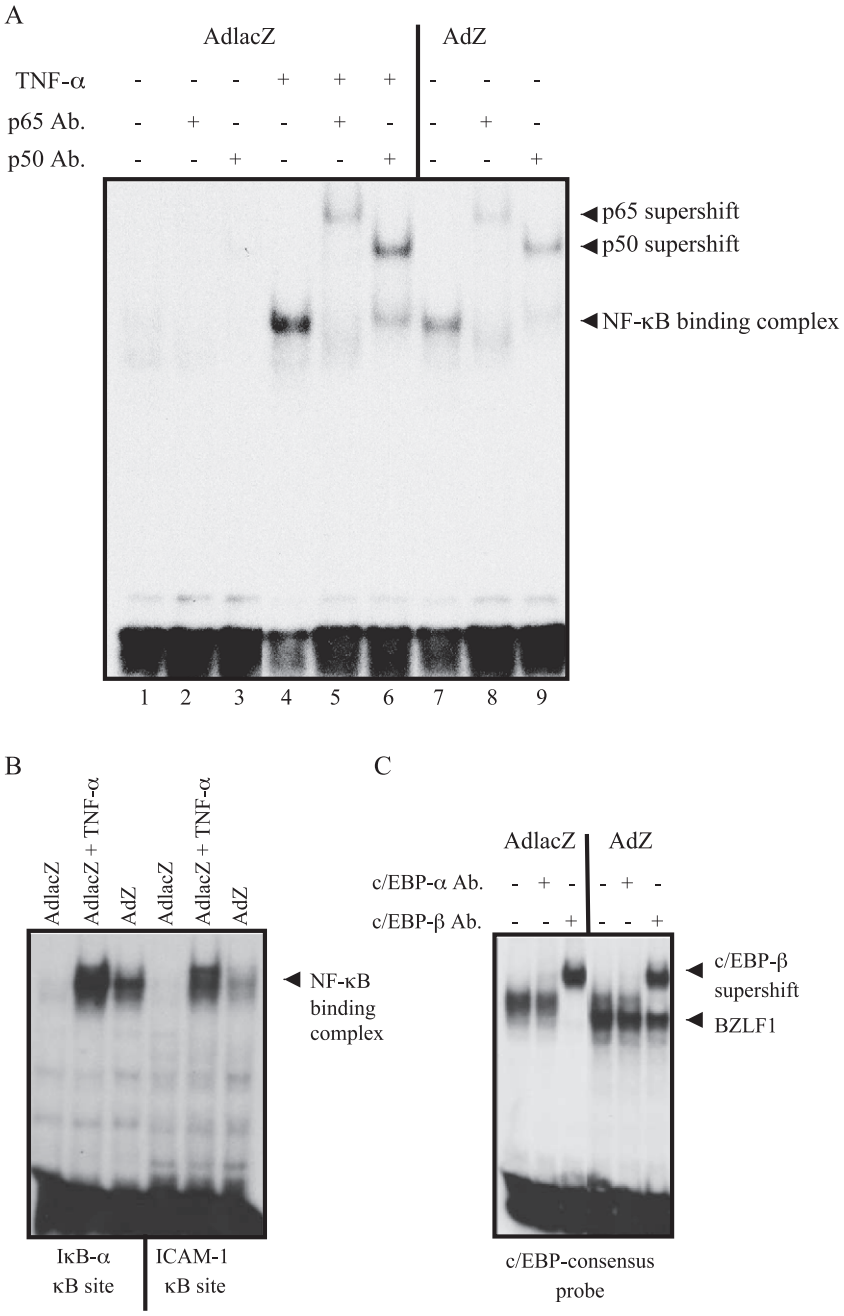

Fig. 9. BZLF1 induces nuclear NFκB DNA binding. HeLa cells were infected with AdlacZ or AdZ (moi 30). At 48 h post-infection, some groups were treated with 10 ng/ml TNF-α for 30 min. Ten micrograms of nuclear extract were analyzed by electrophoretic mobility shift assay using radiolabeled probes containing the NF-κB binding site from the major histocompatibility complex I promoter (A), the IκB-α promoter (B), or the ICAM-1 promoter (B). Extracts were also analyzed using a radiolabeled probe containing the c/EBP consensus site (C). Antibody supershifts were performed with anti-p65 and anti-p50 antibodies (A) as well as anti-c/EBP-α and anti-c/EBP-β antibodies (C).

greatly attenuated by overexpression of p65. Consistent with this, the infectious viral titer of the BZLF1-transfected cell supernatants (determined by quantitating the number of GFP-positive Raji B cells derived from each supernatant) was also dramatically reduced by p65 overexpression (Fig. 11B). These results confirm that p65 inhibits BZLF1 transactivation function and further demonstrate that p65 inhibits the complete EBV lytic replication cycle.

Discussion

NF-κB is a key mediator of the immune response and is utilized by the host to initiate a rapid cellular response to a wide range of stimuli. Target genes include cytokines, chemokines, receptors required for immune recognition, proteins involved in antigen presentation, and adhesion receptors (Ghosh et al., 1998). Thus, it is an attractive target to viral pathogens (Hiscott et al., 2001). In this report, we

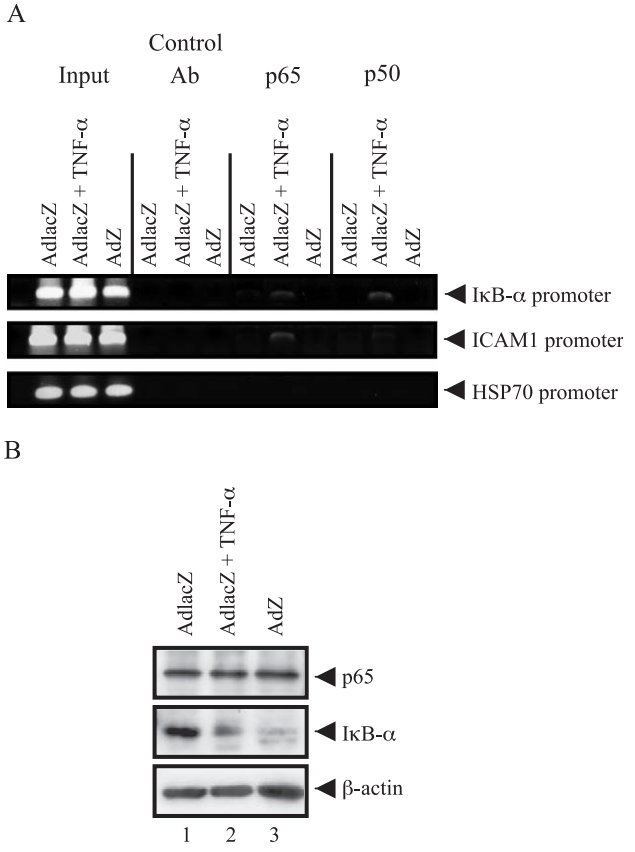

Fig. 10. BZLF1 prevents NF-κB recruitment to the IκB-α and ICAM-1 promoters in vivo. (A) HeLa cells were infected with AdlacZ or AdZ (moi 30). Following stimulation of some groups with 10 ng/ml TNF-α for 30 min, chromatin immunoprecipitation using anti-p65, anti-p50, and control rabbit IgG antibodies was performed to analyze the recruitment of NF-κB to the IκB-α, ICAM-1, and HSP-70 promoters. (B) Immunoblot analysis of protein extracts from (A) for p65, IκB-α, and β-actin.

show that there are multiple and complex interactions between BZLF1 and NF-κB. We present evidence that the EBV immediate–early protein, BZLF1, inhibits gene expression regulated by the NF-κB family member p65. This ability of BZLF1 to inhibit NF-κB transcriptional function may protect the lytic form of EBV infection from the multiple immune-enhancing roles of NF-κB. Additionally, we demonstrate that p65 overexpression inhibits the complete EBV lytic replication cycle. Thus, cells that express a high level of active NF-κB may more easily enter the latent (versus lytic) form of EBV infection following primary infection with this virus. Perhaps most surprisingly, we find that BZLF1 expression results in efficient translocation of NF-κB to the nucleus, presumably due to BZLF1-mediated inhibition of IκB-α expression. Nevertheless, our evidence suggests that this nuclear NF-κB cannot actually activate cellular genes in the presence of BZLF1. Instead, NF-κB nuclear translocation may serve to negatively autoregulate BZLF1 transcriptional activity and thus promote viral latency. In addition, the ability of BZLF1 to enhance translocation of NF-κB to the nucleus may enhance its direct association with p65, allowing it to inhibit p65 transcriptional function.

The BZLF1-mediated induction of NF-κB translocation to the nucleus is comparable to that which is found in the nucleus of HeLa cells following stimulation with TNF-α. Our data suggest that the nuclear translocation of NF-κB in BZLF1-expressing cells, similar to the effect of TNF-α, results from a dramatic decrease in the cellular level of IκB-α protein. In the absence of IκB-α, NF-κB is not retained in the cytoplasm and instead translocates to the nucleus. However, both the mechanism by which BZLF1 induces a decrease in IκB-α expression, as well as the downstream consequences of this effect, are very different from the effects of TNF-α. Whereas TNF-α induces phosphorylation and subsequent destabilization of the IκB-α protein (Karin and Ben-Neriah, 2000), BZLF1 does not appear to affect IκB-α protein stability but instead reduces IκB-α transcription. In addition, whereas the nuclear NF-κB induced by TNF-α treatment results in increased transcription of NF-κB responsive cellular genes, including the IκB-α gene (Ghosh et al., 1998), we found no evidence that the nuclear NF-κB induced by BZLF1 activated either the ICAM-1 or IκB-α genes in the context of the cellular genome. We conclude that the nuclear NF-κB induced by BZLF1 expression is unable to activate transcription of at least a subset of NF-κB target genes in the context of the intact cellular genome.

The exact mechanism by which BZLF1 prevents NF-κB-dependent transcription of cellular genes is not totally clear. Because BZLF1 and p65 physically associate (Gutsch et al., 1994), one possibility is that the interaction between BZLF1 and p65 inhibits p65 binding to DNA. However, the EMSA experiments in Fig. 9 indicate that nuclear NF-κB binding several probes is actually increased by BZLF1 (due to translocation of NF-κB to the nucleus) and thus do not support this hypothesis. It is possible that the p65/BZLF1 complex cannot withstand the EMSA conditions, thereby obscuring an effect of BZLF1 on NF-κB binding. However, our finding that BZLF1 increases the ability of transfected p65 to activate the IκB-α promoter linked to a reporter gene (Fig. 1B) suggests that nuclear NF-κB binds to the IκB-α promoter in the presence of BZLF1 in at least some circumstances. Nevertheless, the results of chromatin immunoprecipitation analysis suggest that NF-κB cannot be efficiently recruited to either the IκB-α or ICAM-1 promoter in the context of the intact cellular genome in BZLF1-expressing cells. Thus, our data suggest that an epigenetic modification of the IκB-α promoter that occurs in the cellular genome, such as histone deacetylation or methylation, but is not adequately modeled using plasmid promoter constructs, is required to observe BZLF1 reduction of NF-κB binding to the IκB-α promoter.

Interestingly, BZLF1 interacts directly with the histone acetylase protein, CBP, and the interaction between CBP and p65 also enhances p65 transcriptional function (Gerrit-

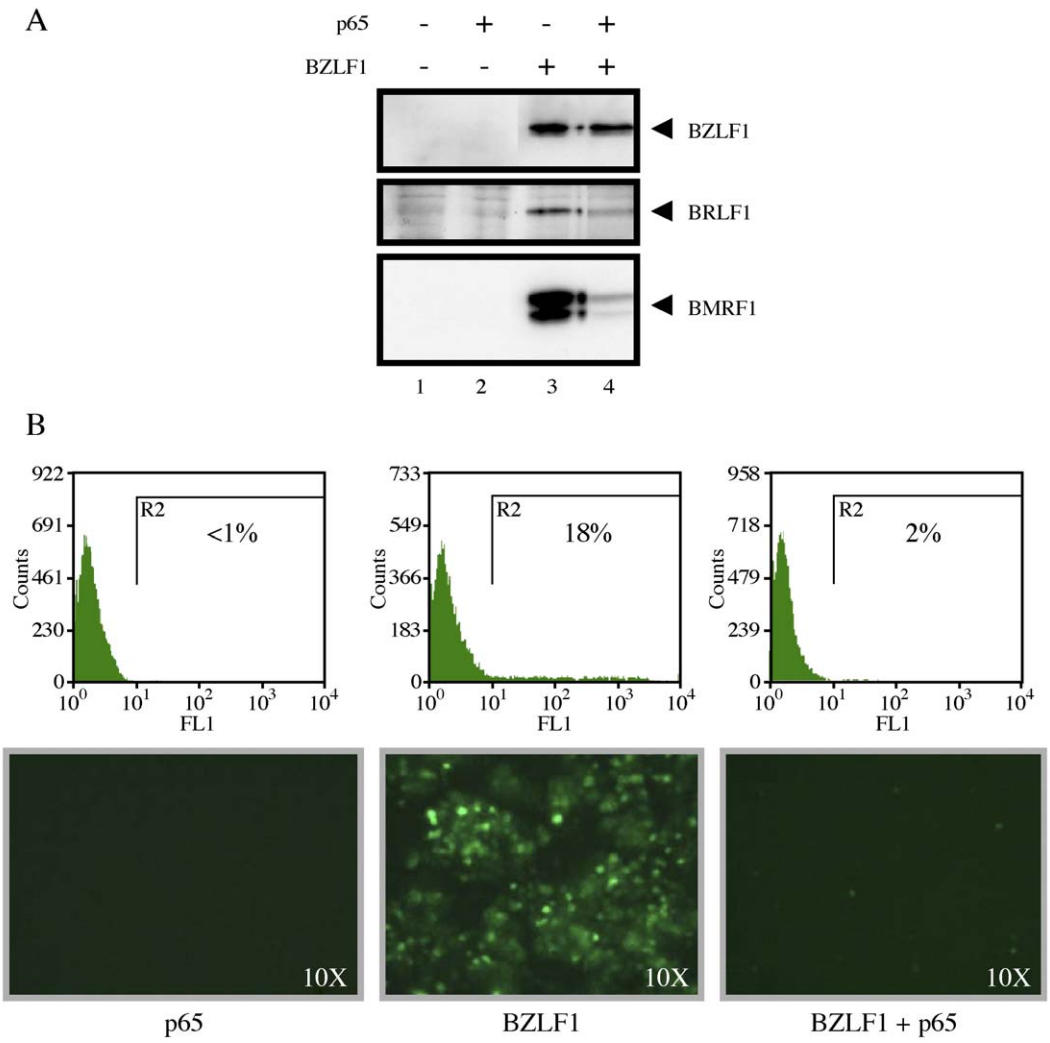

Fig. 11. p65 inhibits induction of the EBV lytic cycle by BZLF1. (A) 293-EBV cells were transfected with expression vectors encoding p65 and BZLF1 as indicated. At 48 h post-transfection, cells were harvested and analyzed by immunoblot analysis for BZLF1 (upper panel), BRLF1 (middle panel), and BMRF1 (lower panel). (B) Raji cells were incubated with filtered cell supernatants from transfected 293-EBV cells (A). After 72 h of incubation, Raji cells were analyzed for GFP expression using an inverted immunofluorescent microscope (lower panel), or the percentage of GFP-expressing cells was determined by FACS analysis (upper panel).

sen et al., 1997). Thus, BZLF1 possibly inhibits p65 transcriptional function, at least partially, by competing for limiting quantities of CBP in the host cell. Our finding that CBP overexpression did not reverse the inhibitory effect of BZLF1 on p65 argues against this mechanism, however. In addition, mutational analysis showed that neither the BZLF1 transactivation domain (which is required for interaction with CBP) (Adamson and Kenney, 1999; Zerby et al., 1999) nor BZLF1 DNA binding are required for inhibition of p65-mediated gene expression by BZLF1. These results suggest that neither the direct interaction between BZLF1 and CBP, nor the ability of BZLF1 to function as a transcription factor, is required for inhibition of p65 function.

The ability of BZLF1 to inhibit NF-κB responsive gene expression in the host cell may play an important role in protecting the virus from the host immune response during the lytic form of infection. Consistent with this, Keating and colleagues recently reported that the cell surface expression of three different NF-κB activated gene products, MHC class I (Baldwin and Sharp, 1988; Israel et al., 1987), CD40 (Krzesz et al., 1999; Wagner et al., 2002), and ICAM-1 (Roebuck and Finnegan, 1999), is reduced on the surface of BZLF1 (+) lymphoblastoid cells (Keating et al., 2002). Thus, the level of BZLF1 expressed during infection of B cells in the context of the intact virus appears to be sufficient to inhibit expression of NF-κB-dependent gene products. These results, together with this report, strongly support the hypothesis that BZLF1 inhibits NF-κB-mediated gene expression, which may be an important mechanism utilized by the virus to evade host immune and inflammatory responses.

In addition to the effects of BZLF1 on the NF-κB pathway, the EBV latent membrane protein 1 (LMP1) is a transmembrane protein that constitutively activates NF-κB

(Hammarskjold and Simurda, 1992; Laherty et al., 1992; Paine et al., 1995). Interestingly, LMP1 impairs induction of the EBV lytic cycle (Adler et al., 2002; Prince et al., 2003), and one mechanism of LMP1-mediated inhibition of the lytic cycle is via NF-κB activation, as expression of a dominant-negative IκB-α abolished this activity (Prince et al., 2003). Additionally, activated CD40, an inducer of NF-κB activation, also inhibits induction of the EBV lytic cycle (Adler et al., 2002). Taken together, these data demonstrate that NF-κB is an important negative regulator of the EBV lytic cycle.

It is well established that NF-κB induces several genes with anti-apoptotic function (Ghosh et al., 1998). Thus, a potential negative consequence for the virus in regard to blocking NF-κB function would be the tendency to promote apoptosis, which might decrease the efficiency of viral replication. However, because EBV encodes several different lytic gene products, including a *bcl-2* homologue, that have anti-apoptotic function, these gene products likely inhibit cellular apoptosis even in the absence of functional NF-κB (Kieff and Rickinson, 2001). Additionally, our group has recently reported that BZLF1 expression inhibits cellular responses to TNF-α, including apoptosis, by down-regulating TNF-R1 expression (Morrison et al., 2004).

There is increasing evidence that NF-κB plays a critical role in the regulation of gammaherpesviruses. Our findings demonstrate that p65 inhibits lytic viral gene expression and the production of new EBV virions when co-transfected with a BZLF1 expression plasmid into latently infected 293 cells. These results suggest that cells that have a high level of active NF-κB at the time of primary EBV infection may be more prone to entering the latent form of infection than cells that have little or no active NF-κB. Similar to results reported here, Brown et al. (2003) found that overexpression of p65 inhibited lytic viral gene expression and release of progeny virus in cells infected with murine gammaherpesvirus-68 (MHV-68). Additionally, treatment of at least one EBV-positive Burkitt's lymphoma line (P3J-HR1), and two different KSHV-positive primary effusion lymphoma lines (BCBL1 and KS-1), with the NF-κB inhibitor Bay11-7082, induced lytic viral gene expression, suggesting that endogenous NF-κB actively suppresses the EBV and KSHV lytic cycle (Brown et al., 2003). However, we have not found that NF-κB inhibitors are sufficient to induce lytic gene expression in EBV-transformed (lymphoblastoid) B-cell lines, indicating that additional factors are also important for maintaining viral latency in these cells (unpublished data).

Interestingly, herpes simplex virus-1 (HSV-1) infection of C33 cells was also shown to result in decreased expression of IκBs and increased nuclear NF-κB (Patel et al., 1998). HSV-1-induced NF-κB nuclear translocation was shown to require expression of the HSV-1 IE proteins ICP4 and ICP27 (Patel et al., 1998). Similar to our findings, the nuclear NF-κB translocation resulting from HSV-1 infection did not appear to result in an induction of NF-κB-dependent gene expression (Patel et al., 1998). However, in contrast to the evidence suggesting that NF-κB negatively regulates the lytic replication cycle of gammaherpesviruses, increased nuclear NF-κB enhanced HSV-1 replication (Patel et al., 1998).

In summary, we have demonstrated that BZLF1 inhibits gene expression induced by NF-κB; likewise, NF-κB inhibits the EBV lytic replication cycle induced by BZLF1. Somewhat surprisingly, we have demonstrated that BZLF1 translocates NF-κB to the nucleus, although the NF-κB in the nucleus does not appear to be functional.

Materials and methods

Cell lines

The HeLa line, a cervical carcinoma cell line, and A549 cells were maintained in Dulbecco's modified Eagle's medium H supplemented with 10% fetal bovine serum (FBS). 293-EBV cells (a gift from Henri–Jacques Delecluse) were maintained in Dulbecco's modified Eagle's medium H supplemented with 10% FBS. The Raji cell line, a Burkitt's lymphoma cell line, was maintained in RPMI 1640 medium supplemented with 10% FBS. All culture media contained penicillin (100 U/ml) and streptomycin (100 μg/ml). All cell lines were cultured at 37 °C in a humidified atmosphere containing 5% CO_2.

Adenovirus construction and infection

The EBV IE gene *BZLF1* and the control *lacZ* gene, under control of the IE CMV promoter, were inserted via cre-loxP-mediated recombination into an adenovirus type 5 derivative lacking the E1 and E3 genes to create adenovirus-LacZ (AdlacZ) and adenovirus-BZLF1 (AdZ), as previously described (Westphal et al., 1999). Recombinant virus stocks were confirmed devoid of wild-type virus by plaque assay and E1A Western blot. Cells were infected with no adenovirus (mock infection), AdlacZ, or AdZ at the multiplicity of infection (moi) indicated.

Plasmid construction

Plasmids pSG5-Zwt and pSG5-ZA185K were constructed by subcloning wild-type BZLF1 cDNA or A185K BZLF1 cDNA (containing a point mutation that alters amino acid 185 from alanine to lysine) from the pHD1013 vector into the *Eco*R1 site of pSG5 (Stratagene) (Morrison et al., 2004). Plasmid pSG5-ZΔTA was generated by *Hin*dIII cleavage of pSG5-Zwt followed by religation, producing an in-frame deletion of amino acids 24–86 (Morrison et al., 2004). Plasmid pSG5-ZΔDIM was generated by subcloning BZLF1 cDNA containing a

deletion of the dimerization domain (DIM) from amino acid 196 to amino acid 228 (a gift from Alain Sergeant) from the pKSV vector into the *Eco*R1 site of pSG5 (Stratagene). The FLAG-tagged p65 expression vector (a gift from Albert Baldwin) encodes p65 cDNA under control of the CMV promoter (Stein et al., 1993). The HA-CBP expression vector contains the CBP cDNA downstream of the CMV immediate–early promoter (a gift from Jenny P.-Y. Ting). The pSEAP-NFκB reporter vector contains four copies of the NFκB consensus sequence and was obtained from Clontech. The reporter plasmid pSEAP-IκB-α was constructed by PCR amplification of a DNA fragment corresponding to the −357/+ 68 region of the IκB-α promoter (relative to the transcription start site) (Algarte et al., 1999; Ito et al., 1994; Le Bail et al., 1993) using oligonucleotide primers to allow for addition of a 5′ *Nhe*I restriction site and a 3′ *Bgl*II restriction site. The PCR product was cloned upstream of the gene encoding secreted alkaline phosphatase (SEAP) by cleavage with *Nhe*I and *Bgl*II and ligation into *Nhe*I/*Bgl*II-digested pSEAP2-Basic (Clontech), which lacks eukaryotic promoter and enhancer sequences.

DNA transfection

Cell lines were transfected using FuGENE 6 transfection reagent (Roche) according to the manufacturer's instructions. Briefly, cells were seeded into six-well plates 1 day before transfection. One microgram of plasmid DNA was combined with 3 μl FuGENE 6 transfection reagent in OptiMEM (Invitrogen) and added directly to cell cultures. For reporter assays, 500 ng of each construct was transfected. Transfection of pSEAP2-Basic with effector plasmids was included as a control in each experiment. Cells were incubated with transfection complexes for 5 h.

Reporter assays

Secreted alkaline phosphatase (SEAP) activity was measured in the supernatant of transfected cell cultures at 48 h post-transfection using a GreatEscAPe™ SEAP chemiluminescent detection kit (CLONTECH) according to the manufacturer's protocol.

Immunoblotting

Cells were lysed in RIPA lysis buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 1% Nonidet P-40, 0.5% deoxycholate, 0.1% sodium dodecyl sulfate [SDS] supplemented with complete protease inhibitor cocktail [Roche]). Protein (30–50 μg) was diluted in an equal volume of 2× SDS sample buffer, and SDS-polyacrylamide gel electrophoresis was performed. Proteins were transferred overnight onto nitrocellulose membranes (Osmonics). Membranes were blocked in 1× phosphate-buffered saline (PBS)/5% milk/0.1% Tween 20 and incubated in primary antibody for 1–2 h at room temperature (ICAM-1 [1:500], p65 [1:200], IκB-α[1:1000], CBP [1:200] and p53 [1:200] from Santa Cruz Biotechnology; β-actin [1:5000] and anti-FLAG [1:1000] from Sigma; phospho-serine 536 of p65 [1:1000] from Cell Signaling Technology; BMRF1 [1:100] from Capricorn, BZLF1 [1:75] from Dako and BZLF1 [1:500] from Argene). Membranes were washed in PBS–0.1% Tween 20 and incubated with secondary antibody for 1 h at room temperature (goat anti-rabbit–horseradish peroxidase [HRP] or goat anti-mouse–HRP [both 1:10,000] from Promega). Following washing, proteins were visualized by enhanced chemiluminescence (Amersham) according to the manufacturer's instructions. Immunoblots were quantitated using ImageJ v1.32 software (NIH).

Chromatin immunoprecipitation assays

HeLa cells (with or without TNF-α stimulation) were cross-linked for 10 min at room temperature by adding formaldehyde (37% from Sigma) directly to the culture medium to a final concentration of 1%. Cross-linking was stopped by the addition of glycine to a final concentration of 125 mM for 5 min. Cells were washed 2× with ice-cold 1× PBS, scraped into 15-ml conical tubes, and centrifuged for 10 min at 4 °C. Chromatin immunoprecipitations were performed following a modification of the Chromatin Immunoprecipitation Assay Kit protocol (Upstate). Briefly, cells were lysed in 100 μl ChIP lysis buffer (50 mM Tris, at pH 8.1, 10 mM EDTA, 1% SDS) supplemented with complete protease inhibitors (Roche). Following dilution in 500 μl ChIP dilution buffer (16.7 mM Tris, pH 8.1, 150 mM NaCl, 1.2 mM EDTA, 1.1% Triton X-100, 0.01% SDS), chromatin was sheared by sonication (power setting 3) with a Misonix XL-2020 sonicator (1× 10-s burst, 2× 35-s pulse [1.0 s ON, 0.8 s off], 1× 10-s burst). Samples were centrifuged to pellet debris and diluted 10× in ChIP dilution buffer. Samples were precleared with 60 μl of a 50% slurry of salmon sperm/protein A agarose beads for 1 h at 4 °C and immunoprecipitations were carried out overnight at 4 °C with 2 μg each of anti-p65 (Santa Cruz), anti-p50 (Santa Cruz), and purified rabbit IgG from normal serum (Sigma). Immune complexes were collected with 60 μl of salmon sperm/protein A agarose beads for 2 h and washed 1× for 15 min in low salt buffer, high salt buffer, LiCl buffer, and 2× for 5 min in TE. Immune complexes were eluted in 1% SDS/0.1 M Na HCO_3 and cross-links were reversed by heating at 65 °C for 5–6 h. Following proteinase K digestion (45 °C for 1 h), DNA was phenol–chloroform extracted once, chloroform extracted once, and ethanol precipitated. Pellets were resuspended in 30 μl TE and one-tenth of the immunoprecipitated DNA was used in each PCR. The human HSP70 promoter was amplified with the PCR primer pairs 5′-GGATCCAGTGTTCCGTTTCC-3′ and 5′-GTCAAACACGGTGTTCTGCG-3′ (Nis-

sen and Yamamoto, 2000). The human IκB-α promoter was amplified with the PCR primer pairs 5′-GACGACCCCAATTCAAATCG-3′ and 5′-TCAGGCTCGGGGAATTTCC-3′ (Saccani et al., 2002). The human ICAM-1 promoter was amplified with the PCR primer pairs 5′-ACCTTAGCGCGGTGTAGACC-3′ and 5′-CTCCGGAACAAATGCTGC-3′ (Nissen and Yamamoto, 2000).

Electrophoretic mobility shift assays (EMSA)

Nuclear extracts were prepared using a modification of the Dignam method (Dignam et al., 1983). Briefly, cells were resuspended in buffer A (20 mM HEPES, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM dithiothreitol, and complete protease inhibitor) with 1% NP-40. Crude nuclei were pelleted at low speed, and the cytosolic fractions were extracted. Nuclei were purified over an OptiPrep gradient with 25%, 30%, and 35% layers and then lysed with NE buffer (20 mM Tris–HCl, 400 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 25% glycerol, and complete protease inhibitors). Oligonucleotides containing the NF-κB binding site from the human MHC class I promoter (CAGGGCTG*GGGATTCCCC*ATCTCCACAGTTTCACTTC) (a gift from Albert Baldwin), the IκB-α promoter (GCTT-*GGAAATTCCCC*GAGCCT), and the ICAM-1 promoter (TTAGCTT*GGAAATTCC*GGAGCTG), as well as oligonucleotides containing the C/EBP consensus site (CTAGCTGCAGA*TTGCGCAAT*CTGCAG), were labeled with [α-^{32}P] dCTP using the Klenow fragment of DNA polymerase I (Roche). Binding reactions contained 5–10 μg nuclear extract and 20,000 cpm radiolabeled oligonucleotide in 10 mM Tris, pH 7.6, 10% glycerol, 1 mM DTT, 0.5 mM EDTA, and 2 μg dIdC. For antibody supershift experiments, 1 μl of antibody was incubated with nuclear extract for 30 min before addition of radiolabeled probe. Binding reactions were electrophoresed in 5% nondenaturing polyacrylamide gels in 1× Tris–Glycine–EDTA buffer. Gels were dried and protein–DNA complexes were visualized by autoradiography.

Northern blot analysis

Twenty micrograms of total RNA, purified using the Qiagen RNeasy Mini Kit as specified by the manufacturer, was separated on a 1% agarose/formaldehyde gel and transferred to nylon membranes (Schleicher and Schuell). Membranes were incubated for 25 min at 68 °C in QuikHyb hybridization solution (Stratagene), and radiolabeled probes were melted, mixed with 100 μl of 10 mg/ml calf thymus DNA, and hybridized to membranes overnight at 68 °C. After hybridization, membranes were washed twice in 2× SSC/0.1% SDS at room temperature and once in 0.2× SSC/0.1% SDS for 30 min at 60 °C. The IκB-α (a gift from Albert Baldwin) and GAPDH (Ambion) DNA probes were radiolabeled with ^{32}P by random priming using an oligolabeling kit (Amersham).

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References

Adamson, A.L., Kenney, S., 1999. The Epstein–Barr virus BZLF1 protein interacts physically and functionally with the histone acetylase CREB-binding protein. J. Virol. 73 (8), 6551–6558.

Adler, B., Schaadt, E., Kempkes, B., Zimber-Strobl, U., Baier, B., Bornkamm, G.W., 2002. Control of Epstein–Barr virus reactivation by activated CD40 and viral latent membrane protein 1. Proc. Natl. Acad. Sci. U.S.A. 99 (1), 437–442.

Algarte, M., Kwon, H., Genin, P., Hiscott, J., 1999. Identification by in vivo genomic footprinting of a transcriptional switch containing NF-kappaB and Sp1 that regulates the IkappaBalpha promoter. Mol. Cell. Biol. 19 (9), 6140–6153.

Arenzana-Seisdedos, F., Thompson, J., Rodriguez, M.S., Bachelerie, F., Thomas, D., Hay, R.T., 1995. Inducible nuclear expression of newly synthesized I kappa B alpha negatively regulates DNA-binding and transcriptional activities of NF-kappa B. Mol. Cell. Biol. 15 (5), 2689–2696.

Baldwin Jr., A.S., 2001. Series introduction: the transcription factor NF-kappaB and human disease. J. Clin. Invest. 107 (1), 3–6.

Baldwin Jr., A.S., Sharp, P.A., 1988. Two transcription factors, NF-kappa B and H2TF1, interact with a single regulatory sequence in the class I major histocompatibility complex promoter. Proc. Natl. Acad. Sci. U.S.A. 85 (3), 723–727.

Brown, K., Park, S., Kanno, T., Franzoso, G., Siebenlist, U., 1993. Mutual regulation of the transcriptional activator NF-kappa B and its inhibitor, I kappa B-alpha. Proc. Natl. Acad. Sci. U.S.A. 90 (6), 2532–2536.

Brown, H.J., Song, M.J., Deng, H., Wu, T.T., Cheng, G., Sun, R., 2003. NF-kappaB inhibits gammaherpesvirus lytic replication. J. Virol. 77 (15), 8532–8540.

Chang, Y.N., Dong, D.L., Hayward, G.S., Hayward, S.D., 1990. The Epstein–Barr virus Zta transactivator: a member of the bZIP family with unique DNA-binding specificity and a dimerization domain that lacks the characteristic heptad leucine zipper motif. J. Virol. 64 (7), 3358–3369.

Chen, C.J., Deng, Z., Kim, A.Y., Blobel, G.A., Lieberman, P.M., 2001. Stimulation of CREB binding protein nucleosomal histone acetyltransferase activity by a class of transcriptional activators. Mol. Cell. Biol. 21 (2), 476–487.

Chevallier-Greco, A., Manet, E., Chavrier, P., Mosnier, C., Daillie, J., Sergeant, A., 1986. Both Epstein–Barr virus (EBV)-encoded *trans*-acting factors, EB1 and EB2, are required to activate transcription from an EBV early promoter. EMBO J. 5 (12), 3243–3249.

Chiao, P.J., Miyamoto, S., Verma, I.M., 1994. Autoregulation of I kappa B alpha activity. Proc. Natl. Acad. Sci. U.S.A. 91 (1), 28–32.

Countryman, J., Miller, G., 1985. Activation of expression of latent Epstein–Barr herpesvirus after gene transfer with a small cloned subfragment of heterogeneous viral DNA. Proc. Natl. Acad. Sci. U.S.A. 82 (12), 4085–4089.

Deng, Z., Chen, C.J., Chamberlin, M., Lu, F., Blobel, G.A., Speicher, D., Cirillo, L.A., Zaret, K.S., Lieberman, P.M., 2003. The CBP bromodomain and nucleosome targeting are required for Zta-directed nucleosome acetylation and transcription activation. Mol. Cell. Biol. 23 (8), 2633–2644.

DiDonato, J.A., Hayakawa, M., Rothwarf, D.M., Zandi, E., Karin, M., 1997. A cytokine-responsive IkappaB kinase that activates the transcription factor NF-kappaB. Nature 388 (6642), 548–554.

Dignam, J.D., Lebovitz, R.M., Roeder, R.G., 1983. Accurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei. Nucleic Acids Res. 11 (5), 1475–1489.

Dreyfus, D.H., Nagasawa, M., Pratt, J.C., Kelleher, C.A., Gelfand, E.W., 1999. Inactivation of NF-kappaB by EBV BZLF-1-encoded ZEBRA protein in human T cells. J. Immunol. 163 (11), 6261–6268.

Farrell, P.J., Rowe, D.T., Rooney, C.M., Kouzarides, T., 1989. Epstein–Barr virus BZLF1 *trans*-activator specifically binds to a consensus AP-1 site and is related to c-fos. EMBO J. 8 (1), 127–132.

Feederle, R., Kost, M., Baumann, M., Janz, A., Drouet, E., Hammerschmidt, W., Delecluse, H.J., 2000. The Epstein–Barr virus lytic program is controlled by the co-operative functions of two transactivators. EMBO J. 19 (12), 3080–3089.

Flemington, E., Speck, S.H., 1990. Autoregulation of Epstein–Barr virus putative lytic switch gene BZLF1. J. Virol. 64 (3), 1227–1232.

Flemington, E.K., Borras, A.M., Lytle, J.P., Speck, S.H., 1992. Characterization of the Epstein–Barr virus BZLF1 protein transactivation domain. J. Virol. 66 (2), 922–929.

Flemington, E.K., Lytle, J.P., Cayrol, C., Borras, A.M., Speck, S.H., 1994. DNA-binding-defective mutants of the Epstein–Barr virus lytic switch activator Zta transactivate with altered specificities. Mol. Cell. Biol. 14 (5), 3041–3052.

Gerritsen, M.E., Williams, A.J., Neish, A.S., Moore, S., Shi, Y., Collins, T., 1997. CREB-binding protein/p300 are transcriptional coactivators of p65. Proc. Natl. Acad. Sci. U.S.A. 94 (7), 2927–2932.

Ghosh, S., Karin, M., 2002. Missing pieces in the NF-kappaB puzzle. Cell 109, S81–S96. (Suppl).

Ghosh, S., May, M.J., Kopp, E.B., 1998. NF-kappa B and Rel proteins: evolutionarily conserved mediators of immune responses. Annu. Rev. Immunol. 16, 225–260.

Gutsch, D.E., Holley-Guthrie, E.A., Zhang, Q., Stein, B., Blanar, M.A., Baldwin, A.S., Kenney, S.C., 1994. The bZIP transactivator of Epstein–Barr virus, BZLF1, functionally and physically interacts with the p65 subunit of NF-kappa B. Mol. Cell. Biol. 14 (3), 1939–1948.

Hammarskjold, M.L., Simurda, M.C., 1992. Epstein–Barr virus latent membrane protein transactivates the human immunodeficiency virus type 1 long terminal repeat through induction of NF-kappa B activity. J. Virol. 66 (11), 6496–6501.

Hardwick, J.M., Tse, L., Applegren, N., Nicholas, J., Veliuona, M.A., 1992. The Epstein–Barr virus R transactivator (Rta) contains a complex, potent activation domain with properties different from those of VP16. J. Virol. 66 (9), 5500–5508.

Hiscott, J., Kwon, H., Genin, P., 2001. Hostile takeovers: viral appropriation of the NF-kappaB pathway. J. Clin. Invest. 107 (2), 143–151.

Israel, A., Kimura, A., Kieran, M., Yano, O., Kanellopoulos, J., Le Bail, O., Kourilsky, P., 1987. A common positive *trans*-acting factor binds to enhancer sequences in the promoters of mouse H-2 and beta 2-microglobulin genes. Proc. Natl. Acad. Sci. U.S.A. 84 (9), 2653–2657.

Ito, C.Y., Kazantsev, A.G., Baldwin Jr., A.S., 1994. Three NF-kappa B sites in the I kappa B-alpha promoter are required for induction of gene expression by TNF alpha. Nucleic Acids Res. 22 (18), 3787–3792.

Karin, M., Ben-Neriah, Y., 2000. Phosphorylation meets ubiquitination: the control of NF-[kappa]B activity. Annu. Rev. Immunol. 18, 621–663.

Keating, S., Prince, S., Jones, M., Rowe, M., 2002. The lytic cycle of Epstein–Barr virus is associated with decreased expression of cell surface major histocompatibility complex class I and class II molecules. J. Virol. 76 (16), 8179–8188.

Kieff, E., Rickinson, A.B., 2001. EpsteinBarr virus and its replication. In: Knipe, D.M., Howley, P.M., Griffin, D.E., Martin, M.A., Lamb, R.A., Roizman, B., Straus, S.E. (Eds.) Fields Virology vol. 2. Lippincott Williams & Wilkins, Philadelphia, pp. 2511–2573.

Krzesz, R., Wagner, A.H., Cattaruzza, M., Hecker, M., 1999. Cytokine-inducible CD40 gene expression in vascular smooth muscle cells is mediated by nuclear factor kappaB and signal transducer and activation of transcription-1. FEBS Lett. 453 (1–2), 191–196.

Laherty, C.D., Hu, H.M., Opipari, A.W., Wang, F., Dixit, V.M., 1992. The Epstein–Barr virus LMP1 gene product induces A20 zinc finger protein expression by activating nuclear factor kappa B. J. Biol. Chem. 267 (34), 24157–34160.

Le Bail, O., Schmidt-Ullrich, R., Israel, A., 1993. Promoter analysis of the gene encoding the I kappa B-alpha/MAD3 inhibitor of NF-kappa B: positive regulation by members of the rel/NF-kappa B family. EMBO J. 12 (13), 5043–5049.

Lieberman, P.M., Hardwick, J.M., Hayward, S.D., 1989. Responsiveness of the Epstein–Barr virus *Not*I repeat promoter to the Z transactivator is mediated in a cell-type-specific manner by two independent signal regions. J. Virol. 63 (7), 3040–3050.

Mercurio, F., Zhu, H., Murray, B.W., Shevchenko, A., Bennett, B.L., Li, J., Young, D.B., Barbosa, M., Mann, M., Manning, A., Rao, A., 1997. IKK-1 and IKK-2: cytokine-activated IkappaB kinases essential for NF-kappaB activation. Science 278 (5339), 860–866.

Mogensen, T.H., Paludan, S.R., 2001. Molecular pathways in virus-induced cytokine production. Microbiol. Mol. Biol. Rev. 65 (1), 131–150.

Morrison, T.E., Mauser, A., Klingelhutz, A., Kenney, S.C., 2004. Epstein–Barr virus immediate–early protein BZLF1 inhibits tumor necrosis factor alpha-induced signaling and apoptosis by downregulating tumor necrosis factor receptor 1. J. Virol. 78 (1), 544–549.

Nissen, R.M., Yamamoto, K.R., 2000. The glucocorticoid receptor inhibits NFkappaB by interfering with serine-2 phosphorylation of the RNA polymerase II carboxy-terminal domain. Genes Dev. 14 (18), 2314–2329.

Paine, E., Scheinman, R.I., Baldwin Jr., A.S., Raab-Traub, N., 1995. Expression of LMP1 in epithelial cells leads to the activation of a select subset of NF-kappa B/Rel family proteins. J. Virol. 69 (7), 4572–4576.

Patel, A., Hanson, J., McLean, T.I., Olgiate, J., Hilton, M., Miller, W.E., Bachenheimer, S.L., 1998. Herpes simplex type 1 induction of persistent NF-kappa B nuclear translocation increases the efficiency of virus replication. Virology 247 (2), 212–222.

Prince, S., Keating, S., Fielding, C., Brennan, P., Floettmann, E., Rowe, M., 2003. Latent membrane protein 1 inhibits Epstein–Barr virus lytic cycle induction and progress via different mechanisms. J. Virol. 77 (8), 5000–5007.

Ragoczy, T., Heston, L., Miller, G., 1998. The Epstein–Barr virus Rta protein activates lytic cycle genes and can disrupt latency in B lymphocytes. J. Virol. 72 (10), 7978–7984.

Rickinson, A.B., Kieff, E., 2001. Epstein–Barr Virus. In: Knipe, D.M., Howley, P.M., Griffin, D.E., Martin, M.A., Lamb, R.A., Roizman, B., Straus, S.E. (Eds.,) Fields Virology, vol. 2. Lippincott Williams & Wilkins, Philadelphia, pp. 2575–2627.

Roebuck, K.A., Finnegan, A., 1999. Regulation of intercellular adhesion molecule-1 (CD54) gene expression. J. Leukocyte Biol. 66 (6), 876–888.

Rooney, C.M., Rowe, D.T., Ragot, T., Farrell, P.J., 1989. The spliced BZLF1 gene of Epstein–Barr virus (EBV) transactivates an early EBV promoter and induces the virus productive cycle. J. Virol. 63 (7), 3109–3116.

Saccani, S., Pantano, S., Natoli, G., 2002. p38-Dependent marking of inflammatory genes for increased NF-kappa B recruitment. Nat. Immunol. 3 (1), 69–75.

Santoro, M.G., Rossi, A., Amici, C., 2003. NF-kappaB and virus infection: who controls whom. EMBO J. 22 (11), 2552–2560.

Sizemore, N., Leung, S., Stark, G.R., 1999. Activation of phosphatidylinositol 3-kinase in response to interleukin-1 leads to phosphorylation and activation of the NF-kappaB p65/RelA subunit. Mol. Cell. Biol. 19 (7), 4798–4805.

Sizemore, N., Lerner, N., Dombrowski, N., Sakurai, H., Stark, G.R., 2002. Distinct roles of the Ikappa B kinase alpha and beta subunits in liberating nuclear factor kappa B (NF-kappa B) from Ikappa B and in phosphorylating the p65 subunit of NF-kappa B. J. Biol. Chem. 277 (6), 3863–3869.

Stein, B., Cogswell, P.C., Baldwin Jr., A.S., 1993. Functional and physical associations between NF-kappa B and C/EBP family members: a Rel domain-bZIP interaction. Mol. Cell. Biol. 13 (7), 3964–3974.

Sun, S.C., Ganchi, P.A., Ballard, D.W., Greene, W.C., 1993. NF-kappa B controls expression of inhibitor I kappa B alpha: evidence for an inducible autoregulatory pathway. Science 259 (5103), 912–915.

Takada, K., Shimizu, N., Sakuma, S., Ono, Y., 1986. *trans* activation of the latent Epstein–Barr virus (EBV) genome after transfection of the EBV DNA fragment. J. Virol. 57 (3), 1016–1022.

Urier, G., Buisson, M., Chambard, P., Sergeant, A., 1989. The Epstein–Barr virus early protein EB1 activates transcription from different responsive elements including AP-1 binding sites. EMBO J. 8 (5), 1447–1453.

Wagner, A.H., Gebauer, M., Pollok-Kopp, B., Hecker, M., 2002. Cytokine-inducible CD40 expression in human endothelial cells is mediated by interferon regulatory factor-1. Blood 99 (2), 520–525.

Westphal, E.M., Mauser, A., Swenson, J., Davis, M.G., Talarico, C.L., Kenney, S.C., 1999. Induction of lytic Epstein–Barr virus (EBV) infection in EBV-associated malignancies using adenovirus vectors in vitro and in vivo. Cancer Res. 59 (7), 1485–1491.

Woronicz, J.D., Gao, X., Cao, Z., Rothe, M., Goeddel, D.V., 1997. IkappaB kinase-beta: NF-kappaB activation and complex formation with IkappaB kinase-alpha and NIK. Science 278 (5339), 866–869.

Yang, F., Tang, E., Guan, K., Wang, C.Y., 2003. IKK beta plays an essential role in the phosphorylation of RelA/p65 on serine 536 induced by lipopolysaccharide. J. Immunol. 170 (11), 5630–5635.

Zalani, S., Holley-Guthrie, E., Kenney, S., 1996. Epstein–Barr viral latency is disrupted by the immediate–early BRLF1 protein through a cell-specific mechanism. Proc. Natl. Acad. Sci. U.S.A. 93 (17), 9194–9199.

Zandi, E., Rothwarf, D.M., Delhase, M., Hayakawa, M., Karin, M., 1997. The IkappaB kinase complex (IKK) contains two kinase subunits, IKKalpha and IKKbeta, necessary for IkappaB phosphorylation and NF-kappaB activation. Cell 91 (2), 243–252.

Zerby, D., Chen, C.J., Poon, E., Lee, D., Shiekhattar, R., Lieberman, P.M., 1999. The amino-terminal C/H1 domain of CREB binding protein mediates zta transcriptional activation of latent Epstein–Barr virus. Mol. Cell. Biol. 19 (3), 1617–1626.